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Investigation of Murine Cytomegalovirus Latency and Reactivation in Mice Using Viral Mutants and the Polymerase Chain Reaction

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Studies with 6 ts mutants of mouse cytomegalovirus indicated that mutants tsm1, tsm2, tsm3, and tsm6, like wild-type (*wt*) virus, produced acute infection in mice, became latent, and were reactivated as infectious virus by immunosuppression. Using PCR, all five viruses expressed immediate-early (IE)-1, early (E)-1, and late (L, gB) genes during acute infection in all tissues examined (salivary glands, lung, spleen, liver, kidney, and heart). DNA was present in most tissues during latent infection with all five viruses, but transcription was restricted to the IE-1 gene in the salivary glands of *wt* infected mice only, suggesting true molecular latency rather than low level virus persistence. Similarly, mutant tsm5 expressed all three genes following primary inoculation. Although no detectable virus was produced, tsm5 subsequently entered the latent state as evidenced by DNA detection without RNA transcription indicating that productive infection is not required to initiate latency. This mutant also failed to reactivate from latency, although all three marker genes were expressed in most tissues. In contrast, tsm4 expressed all three marker genes and produced infectious virus during acute infection, then became latent. However, upon immunosuppression to reactivate tsm4, IE-1 and E-1 transcription occurred but neither gB transcription nor infectious virus was detectable in salivary glands, lung, spleen, liver, kidney, heart, or blood. The significance of this with regard to reactivation from latency is discussed.

KEY WORDS: temperature-sensitive mutants, reactivation, transcription

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus, generally causing asymptomatic infection, but it is associated with a number of human diseases, including pneumonitis, gastroenteritis, hepatitis, retinitis, and myocarditis [Alford and Britt, 1993; Ho, 1991]. Populations that are immunologically immature (neonates) or immunosuppressed, either by disease or therapeutically, are at risk of developing CMV-related disease from primary infection or reactivation of latent infection. Reactivation of latent virus is important for life-threatening CMV pneumonitis in transplant recipients, particularly those receiving bone marrow transplants where 15% of such patients develop CMV pneumonia with an 88% case fatality rate [Griffiths and Grundy, 1988]. HCMV is therefore of major clinical significance, as is latency and reactivation, yet the sites of latency and the viral determinants involved are poorly understood.

The host specificity of HCMV has prevented the adoption of an animal model for investigating the pathogenic processes whereby the human virus infects host cells, replicates within them, enters latency, and is reactivated, and such studies are presently impossible in human systems. Murine cytomegalovirus (MCMV) infects mice, inducing a latent infection like HCMV and causing pneumonitis in immunosuppressed mice [Alford and Britt, 1993; Ho, 1991]. Therefore, MCMV has been adopted widely as an animal model for the study of cytomegalovirus infection and reactivation. In addition, the validity of this model for investigations of the molecular mechanisms involved in pathogenicity has been strengthened with recent information on genome organization and sequence data [Buhler et al., 1990; Dallas et al., 1994; Elliott et al., 1991; Keil et al., 1987; Loh et al., 1994; Lyons et al., 1994; Messerle et al., 1991, 1992a,b; Rapp et al., 1992, 1994; Xu et al., 1994].

We reported previously the isolation and phenotypic characterization of 31 temperature-sensitive (ts) mutants of MCMV comprising at least 24 complementation groups and differing in virulence for mice [Akel and Sweet, 1993; Sammons and Sweet, 1989]. Six of these mutants, tsm1-6, have been studied in some detail and

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showed differences in ability to replicate in the lungs and to cause pneumonitis in intranasally inoculated immunosuppressed mice and in their ability to reactivate from the latent state during immunosuppression [Furrarah and Sweet, 1994].

Analysis of MCMV latency and reactivation has not yet entirely resolved whether virus persists in low levels during latency, or whether true molecular latency (absence of infectious virus but presence of viral sequences) exists. It has been argued that expression of the IE-1 gene during latency indicates low level virus persistence since products of the major immediate-early promoter are considered critical for viral replication [Yuhasz et al., 1994]. In contrast, studies using infectivity assays sufficient to detect 1 pfu/organ failed to detect persistent infection during latency [Pollock and Virgin, 1995].

In the present study, the polymerase chain reaction (PCR) and reverse transcription (RT)-PCR of an immediate-early (IE-1), early (E-1) and late (L, gB) gene were used to explore gene transcription during acute infection, latency, and reactivation in mice infected with wild-type (*wt*) virus and mutants tsm1-6. The results suggest that latency does not require continuous low level production of infectious virus since we failed to detect E-1 or gB expression during latent infection with any virus. Indeed, mutant tsm5 became latent but failed to produce infectious virus in mice both during acute infection and following reactivation, suggesting also that a productive infection is not required to initiate the latent state. Interestingly, mutant tsm4, which failed to reactivate from latency yet productively replicated during acute infection, was blocked at gB expression, indicating as suggested recently for Herpes simplex virus (HSV) [Kramer and Coen, 1995] that expression of IE genes is in itself insufficient for reactivation from latency and that a critical threshold level for each gene class needs to be reached for the normal lytic cycle to proceed.

MATERIALS AND METHODS

Viruses and Their Titration

Temperature-sensitive mutants of murine cytomegalovirus, tsm1-tsm6, have been described previously [Akel and Sweet, 1993; Sammons and Sweet, 1989] as have their assay in MEF cells at permissive (33°C) and nonpermissive (39°C or 40°C) temperatures [Akel and Sweet, 1993; Sammons and Sweet, 1989]. The parental *wt* Osborn strain of virus also has previously been described [Sammons and Sweet, 1989] and has now been designated as the K181 (Birmingham) strain. To produce virus stocks, the viruses were passaged twice in 3-week-old CD1 mice and the salivary glands were harvested on each occasion 2–3 weeks after intraperitoneal (i.p.) inoculation of 10^2 pfu of virus. Tissues were homogenized in a small volume of EMEM (Eagle's MEM containing 4% newborn bovine serum, 2 mM L-glutamine, 100 units penicillin per ml, and 100 μg streptomycin per ml) using a Sorvall Omnimixer as described previously [Sammons and Sweet, 1989] to give 10% suspensions. These were clarified by low speed centrifugation (2,500g, 10 min, 4°C), ultrasonicated for 10 sec in a sonicating waterbath, stabilized with a final concentration of 10% DMSO, and stored at −70°C until titrated [Sammons and Sweet, 1989]. Stock viruses prepared in this way contained 8.6×10^6 (*wt*), 2×10^5 (tsm1), 1.3×10^5 (tsm2), 8.6×10^4 (tsm3), 2×10^5 (tsm4), and 1×10^6 (tsm6) pfu/ml. Tsm5, which produced no detectable infectious virus in salivary glands 2–3 weeks after inoculation with 10^2 pfu, was grown as tissue culture stocks by inoculation of MEF cells at a low moi (~0.1 pfu/cell) and harvesting the culture fluid when cells showed >90% cpe. The fluids were sonicated and filtered (0.22 μm) before use to remove multicapsid virions, which are present in tissue culture grown virus but not mouse grown virus [Weiland et al., 1986].

Cells

MEF cells were prepared and maintained as described previously from CD1 mice [Sammons and Sweet, 1989]. The continuous mouse fibroblast cell line 3T3 was kindly donated by Dr. C.W. Dawson (Dept. Cancer Studies, University of Birmingham) and was grown in EMEM with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), sodium bicarbonate (0.11%), and newborn calf serum (10%).

Mice

Specific pathogen-free BALB/c and CD1 mice were supplied by the Biomedical Services Unit (University of Birmingham).

Fifty % Lethal Dose (LD_{50}) Determination

This was determined as previously described [Sammons and Sweet, 1989]. The lethality (pfu/LD_{50}) of each of the viruses were 30, 1,200, 270, 2,700, 2,630, and 144 for wt and mutants tsm1, tsm2, tsm3, tsm4, and tsm6, respectively. Mutant tsm5 was not lethal for mice.

Reactivation of Virus

Groups of 4-week-old BALB/c mice were inoculated i.p. with 1 LD_{50} (as determined for 1-week-old mice) of virus. For each virus, two animals were killed 21 days postinoculation, and the tissues removed and examined by PCR to ensure that each group of animals had been infected. Approximately 7 months later, a further six animals from each group were killed, their tissues removed, and examined similarly to ensure that these animals were no longer shedding virus. The remaining animals were inoculated i.p. with 100 mg/kg cortisone acetate (Sigma) on alternate days for 3 weeks and 0.3 ml of antimouse lymphocyte serum (Sigma) twice weekly for 2 weeks. On days 3, 7, 14, 21, and 28 after initiation of immunosuppression, i.e., after the first injection of cortisone, the animals were killed by overdose of Sagatal (0.1 ml), tissues removed, and examined for virus infectivity and by PCR. The drug doses used for immunosuppression represent a reduction from those used previously [Furrarah and Sweet, 1994], as a pilot study with wt virus indicated a similar level of reactivation with reduced distress to the animals. Tissue from mice with MCMV reactivation often proved toxic for

tissue culture cells, but this was overcome initially by inoculation of undiluted samples into 75 cm^2 flasks where evidence of cpe indicated reactivated virus. It was found later that if the inoculum was removed within 30 min of addition to the cells and the latter were thoroughly washed before the overlay medium was added, the toxicity was removed [Furrarah and Sweet, unpublished observation].

Tissue Homogenisation

Tissue removed from the mice was homogenized and assayed for virus in MEFs following a previously published protocol [Sammons and Sweet, 1989].

Nucleic Acid Isolation From Mouse Tissues for PCR

Whole blood was collected into EDTA to prevent clotting, diluted 1:1 with phosphate-buffered saline, and centrifuged to pellet all cells. DNA was then isolated from the pelleted blood cells and other mouse tissue samples following a standard protocol of SDS lysis and proteinase K digestion followed by phenol/chloroform extraction [Sambrook et al., 1989]. Total RNA was isolated from tissue samples using TriSolv™ (Biogenesis Ltd.), following the protocol supplied. DNA and RNA were quantified subsequently using a UV spectrophotometer.

Polymerase Chain Reaction

Primers were designed from sequence data published previously specifically to amplify sequences from the IE-1 [Keil et al., 1987], E-1 [Buhler et al., 1990] and gB [Rapp et al., 1992] genes of MCMV. The oligonucleotide primers (synthesized by Alta Biosciences, Birmingham University) had the following sequences: IE-1 forward 5′-GATGAGAACCGTGTCTACC-3′, IE-1 reverse 5′-CAACATGTCCTCCAGAGTC-3′; E-1 forward 5′-GTAAGAGATTCGAACTCTGC-3′, E-1 reverse 5′-TGCATTACCATCGACAGTGG-3′; gB forward 5′-CGAAAGAGGATGTCGCTCC-3′, gB reverse 5′-CTCTTGTACGGGTGTCTTCG-3′. Amplification from DNA followed a standard procedure. Briefly, 1 μg DNA in a 100 μl reaction volume (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM each primer, 200 μM each dATP, dCTP, dGTP, and dTTP and two units Taq DNA polymerase (Gibco-BRL)) was amplified over 25 cycles in a Techne PHC-2 temperature cycler with denaturation at 94°C for 30 s, annealing at 50°C for 60 s and extension at 72°C for 120 s. The amplified DNA products were 207, 358, and 400 bp for IE-1, E-1, and gB, respectively. Amplification from an RNA template (RT-PCR) employed the same primers, but using the IE-1 and E-1 specific primers, this resulted in a differently sized amplification product (110 and 266 bp, respectively) as the primers spanned an intronic sequence between exons 1 and 2 for IE-1 and E-1 respectively. The gB gene contained no intronic sequences, and here amplified products from RNA were differentiated from DNA amplified products by ensuring that conventional PCR of extracted RNA without reverse transcriptase produced no products. Total RNA (200 ng) were reverse transcribed in 1 mM Tris-HCl pH 8.3, 5 mM KCl, 1.6 mM $MgCl_2$, 0.005% gelatin and 200 μM dNTPs, and 5 U AMV reverse transcriptase (Pharmacia Biotech) for 1 h at 42°C. The resultant cDNA was then denatured, 2 U Taq polymerase added, and amplified following the conditions described above. All PCR products were electrophoresed through 1.5% agarose to identify the correctly sized amplified product. Furthermore, restriction endonuclease digestion of the products confirmed amplification of the correct sequences.

RESULTS

Validation of PCR Primers

Preliminary experiments showed that the sensitivity of the assay was <10 genome equivalents in a sample of 1 μg total DNA and confirmed the specificity of the primers for MCMV in that no amplification from control DNA isolated from HCMV, Epstein-Barr virus, human papillomavirus type 16, human blood, and MCMV-negative mouse tissue occurred (data not shown). Similar results were obtained with RNA isolated from the same samples and subjected to RT-PCR. In contrast, DNA and RNA isolated from 3T3 cells infected with MCMV yielded the correct size products upon amplification with primers specific for IE-1, E-1, and gB genes (Fig. 1a). Indeed, DNA and RNA were detected by PCR in all tissues removed from a mouse inoculated with *wt* MCMV 14 days previously (Fig. 1b). Endonuclease restriction analysis with *Alu*I, *Ava*I, *Dde*I, *Hin*fI, *Mbo*I, *Sau3*AI, and *Taq*I confirmed the identity of the PCR products amplified with IE-1, E-1, and gB specific primers, respectively (data not shown).

Protocols for Reactivation of Latent Virus

Previously, a standard immunosuppression protocol was used for reactivation of latent virus, which involved daily injection of 100 mg/kg cortisone acetate (CA) for 3 weeks and 0.3 ml antimouse lymphocyte serum (ALS) twice weekly for 2 weeks [Furrarah and Sweet, 1994]. Since this results in some deaths and considerable trauma, additional protocols were investigated in groups of 4 BALB/c mice, originally inoculated at 4 weeks of age with 30 pfu of *wt* virus, treated with one of four different protocols 6 months postinfection to reactivate the virus. As previously, daily injections of CA and twice-weekly injections of ALS (protocol 1; Table I) reactivated virus from all tissues examined, albeit at low level. Mice treated with CA on alternate days and ALS twice-weekly (protocol 2) also reactivated similar levels of virus from all tissues but with considerably less trauma and no deaths (Table I). Further reduction in immunosuppression showed limited reactivation with only low virus titres in some tissues (Table I). PCR and RT-PCR confirmed these results (Table I). In general, for tissues and protocols where virus was reactivated, MCMV DNA and RNA for IE-1 and gB genes could be detected. An anomaly was the inability to detect gB RNA in heart tissue of mice reactivated with protocol 2, even though infectious virus was detected. Interestingly, in most tissues but not all, where reactivated virus could not be detected no

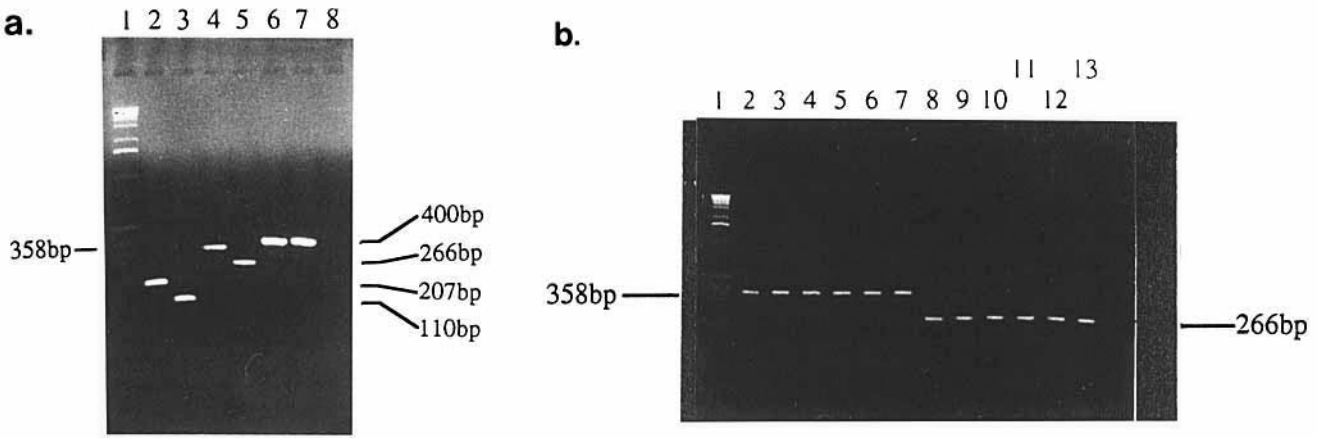

Fig. 1. PCR amplification of MCMV DNA and RNA. Murine cytomegalovirus DNA and RNA was isolated from **(a)** infected 3T3 cells and **(b)** mouse tissue 14 days postinfection with wild-type MCMV. In (a), **lanes 2, 4, and 6** show products amplified from DNA with IE-1, E-1, and gB specific primers, respectively, whereas **lanes 3, 5, and 7** show the corresponding products amplified from RNA: **Lane 8** shows the lack of an amplified product when RNA was amplified with gB specific primers but omitting incubation with reverse transcriptase. In (b), **lanes 2–7** show products amplified from DNA and **lanes 8–13** from RNA extracted from salivary glands (**lanes 2 and 8**), lung (**lanes 3 and 9**), spleen (**lanes 4 and 10**), liver (**5 and 11**), kidney (**lanes 6 and 12**), and heart (**lanes 7 and 13**) using E-1 specific primers. **Lane 1** in both (a) and (b) contains 1Kb DNA ladder (Gibco BRL).

gB RNA could be amplified. Thus protocol 2 was used for all subsequent experiments.

PCR Amplification of MCMV DNA and IE-1, E-1, and gB RNA From Mice Infected With Wt Virus and Mutants Tsm1, Tsm2, Tsm3, and Tsm6

To investigate the viral activity during latency and reactivation, a group of fifty 4-week-old BALB/c mice were inoculated i.p. with 1 LD_{50} (as determined in 1-week-old mice) of wt. Previous investigations had indicated that by 21 days p.i., virus has been cleared from all tissues except salivary glands where virus replication is reaching peak titres, and possibly kidney where virus may persist for up to 19 days p.i [Furrarah and Sweet, 1994]. Thus, 21 days p.i., two mice were sacrificed and salivary glands and kidneys removed for PCR analysis to confirm that the mice were infected and that PCR was able to detect relevant transcriptional activity during acute infection. Amplification of nucleic acid revealed the presence of DNA (Fig. 2) and IE-1, E-1 and gB transcripts in these tissues (Fig. 2). Similar results were obtained for salivary glands from two mice selected from similar groups inoculated with mutants tsm1, tsm2, tsm3, and tsm6 (data not shown). However, gB transcripts were detected only in kidneys of mice inoculated with tsm1; IE-1 and E-1 transcripts were detected in the kidneys of mice infected with mutants tsm2 and tsm3, whereas only IE-1 transcripts were detected in the kidneys of mice infected with tsm6.

Having confirmed that the mice were infected, they were then left for a further 6 months, when six mice were killed to confirm that the viral infection had entered a latent phase, prior to immunosuppression of the mice. As expected, viral culture of the salivary glands removed from all mice proved to be negative in virus assay (data not shown). However, it was possible to amplify MCMV DNA sequences from salivary gland, lung, spleen, and liver from mice inoculated with *wt* (Fig. 2), tsm1, or tsm2 and from spleen and liver from mice inoculated with tsm3 and tsm6. Amplification from RNA sequences coult not be achieved from any tissue, with the exception of IE-1 RNA from the salivary glands of mice inoculated with wt virus (Fig. 2), suggesting that little gene expression was taking place at this time.

As shown previously [Furrarah and Sweet, 1994], immunosuppression reactivated virus from mice latently infected with *wt* virus or mutants tsm1, tsm2, tsm3, and tsm6. For example, in the current experiments titres of 3.3 (±0.2), 2.1 (±0.2), 1.9 (±0.1), and 1.4 (±0.2), $\log_{10}$ pfu/ml were obtained in the salivary glands, lung, kidney, and heart, respectively, of mice infected with *wt* virus and 1.3 (±0.3), 1.4 (±0.1), 0.8 (±0.2), and 0.6 (±0.2) for the same tissues for mice infected with tsm1. However, MCMV DNA and IE gene expression was detected in all mice 3 days postinitiation of immunosuppression (p.i.i.) with all five viruses, even from those tissues that were negative for DNA during latency. With *wt* virus (Fig. 2), expression of the E-1 gene was also detected in blood at this time and by 7 days p.i.i. E-1 and gB gene expression was detected in salivary glands, lung, and spleen as well as blood. This progression from IE through E to L gene expression was delayed in other tissues; E-1 gene expression was detectable at 7 days p.i.i. in liver, but gB gene expression was not evident until 14 days. Similarly, the E-1 and gB genes were not expressed until 14 days p.i.i. in heart, and whereas E-1 gene expression was delayed until 14 days in kidneys, gB gene expression was not detectable until 21 days p.i.i. By the end of the immunosuppression period, all tissues were expressing all three marker genes. A generally similar pattern was observed with mutants tsm1, tsm2, tsm3, and tsm6, with differences in timing of gene expression between viruses and tissues (tsm1 as an example is shown in Fig. 3). A difference was observed in the kid-

TABLE I. Reactivation of Infectious Virus From, and PCR and RT-PCR Analysis of, Various Tissues of Mice Latently Infected With *wt* Virus and Immunosuppressed With Various Protocols*

Protocol[a]	Template	Tissue					
		SG[b]	Spleen	Lung	Liver	Kidney	Heart
1	DNA	+	+	+	+	+	+
	RNA-IE	+	+	+	+	+	+
	RNA-gB	+	+	+	+	+	+
	Mean virus titre (log_{10} pfu/ml ± SE)	3.6 (±0.1)	1.2 (±0.1)	2.2 (±0.1)	1.3 (±0.1)	2.3 (±0.1)	1.4 (±0.1)
2	DNA	+	+	+	+	+	+
	RNA-IE	+	+	+	+	+	+
	RNA-gB	+	+	+	+	+	−
	Mean virus titre (log_{10} pfu/ml ± SE)	3.4 (±0.1)	1.2 (±0.1)	2.1 (±0)	1.0 (±0)	2.1 (±0)	1.4 (±0.1)
3	DNA	+	+	+	+	+	+
	RNA-IE	+	+	+	+	+	−
	RNA-gB	+	−	+	−	−	−
	Mean virus titre (log_{10} pfu/ml ± SE)	1.6 (±0)	<1.0	<1.0	<1.0	<1.0	<1.0
4	DNA	+	+	+	+	+	+
	RNA-IE	+	+	+	+	+	−
	RNA-gB	+	−	+	+	−	−
	Mean virus titre (log_{10} pfu/ml)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

*+, presence of amplified product; −, absence of amplified product from required DNA or RNA template.
[a]Protocol 1: Cortisone acetate (CA) daily for 3 weeks and anti-mouse lymphocyte serum (ALS) twice weekly for 2 weeks. Protocol 2: CA alternate days for 3 weeks and ALS twice weekly for 2 weeks. Protocol 3: CA alternate days for 3 weeks and ALS once weekly for 2 weeks. Protocol 4: CA daily for 3 weeks and ALS once weekly for 2 weeks.
[b]Salivary gland.

neys of tsm3 inoculated mice in which gB expression was evident 21 days p.i.i. in one of two animals but in no animal at times prior to this or at day 28. A similar pattern was seen with tsm6 except that both animals expressed the gB gene at 21 days p.i.i.

PCR Amplification of MCMV DNA and RNA From Mice Inoculated With Mutant Tsm4

Expression of IE-1, E-1 and gB genes in mice acutely infected with mutant *tsm4* was essentially similar to that described above for wt virus and mutants tsm1, tsm2, tsm3, and tsm6. In contrast, mutant tsm4 failed to reactivate from latency (Fig. 4). IE-1 gene expression was detected in all tissues 3 days p.i.i., and E-1 gene expression was evident in blood 7 days p.i.i., in salivary glands, lung, and liver 14 days p.i.i. and in spleen, kidney, and heart 21 days p.i.i., but gB gene expression was not detectable in any tissue at any time point. Indeed, E-1 gene expression was also transient in some tissues being undetectable in kidney and blood by 28 days p.i.i.

PCR Amplification of MCMV DNA and RNA From Mice Inoculated With Mutant Tsm5

The PCR and RT-PCR techniques allowed an investigation of the reason for the lack of replication of tsm5 in mice. To examine this, groups of three mice were inoculated with 3,000 pfu of tsm5 and killed at 3, 7, 10, 14, 21, and 60 days p.i. Although no infectious virus could be detected, MCMV DNA was present in all tissues at all times up to 21 days p.i. (with the exception of heart at 21 days) (Fig. 5). There was also transcriptional activity of all three genes in all tissues, except kidney, at some time, although this was generally restricted to earlier time points.

By 60 days p.i., the virus was latent in salivary glands as judged by the presence of viral DNA, but there was no evidence of viral gene expression in this tissue or of viral DNA or expression in any other tissue (Fig. 5).

Following immunosuppression, infectious virus was not reactivated, but DNA could now be detected in salivary glands, lung, liver, and blood 3 days p.i.i. and in spleen, kidney, and heart 7 days p.i.i. (Fig. 6). In addition, IE, E, and L gene expression occurred in all tissues except kidney, where only IE gene expression was evident, although in liver and heart, L gene expression was transient (Fig. 6).

DISCUSSION

Previously, we showed that our temperature-sensitive mutants exhibited differences in their ability to reactivate from the latent state; in particular, mutants tsm4 and tsm5 did not reactivate using an immunosuppressive protocol that reactivated *wt* virus and mutants tsm1, tsm2, tsm3, and tsm6 and has been shown to induce severe immunosuppression [Shanley et al., 1979]. We used PCR and RT-PCR to examine whether these mutants are incapable of entering the latent state or of being reactivated from it. Three important observations have arisen from these studies. First, all mutants entered the latent state, even tsm5, which did not produce detectable infectious virions at any time following inoculation, suggesting that productive infection is not required for virus to become latent. Second, virus appears

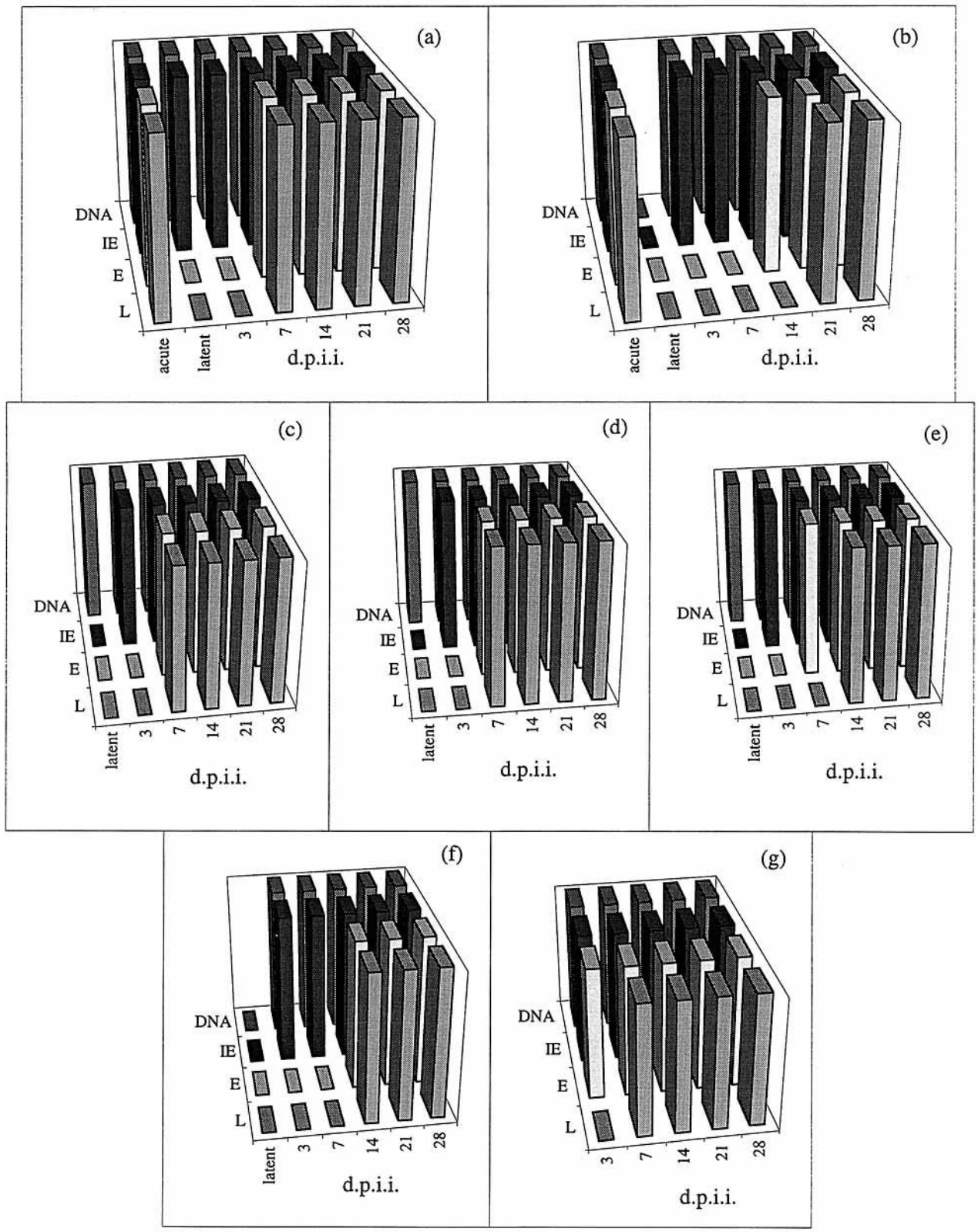

Fig. 2. PCR and RT-PCR analysis of tissues from mice infected with wild-type MCMV and reactivated by immunosuppression. At 21 days postinfection (acute), mice were killed and salivary glands **(a)** and kidneys **(b)** examined for DNA (■) and immediate-early (IE: ■), early (E; ▒), and late (L; ▒) gene expression. Six months later, further mice were examined for MCMV DNA and RNA transcripts (latent) in salivary glands (a), kidney (b), lung **(c),** spleen **(d),** liver **(e),** and heart **(f).** Finally, at 3, 7, 14, 21, and 28 days postinitiation of immunosuppression, mice were killed and the above tissues and blood **(g)** examined similarly. The presence of a column indicates that an amplified product was obtained.

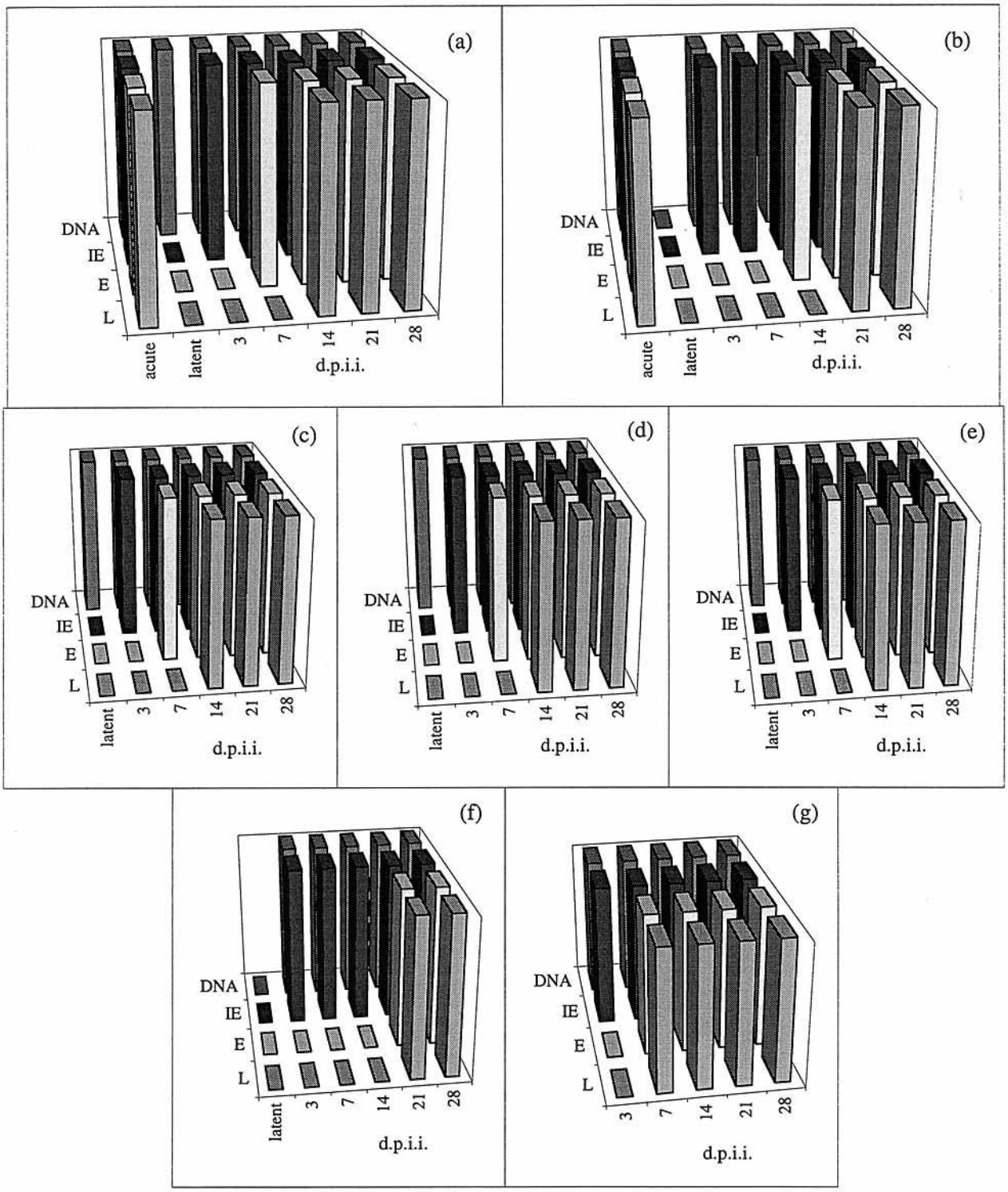

Fig. 3. PCR and RT-PCR analysis of tissues from mice infected with MCMV mutant tsm1 and reactivated by immunosuppression. At 21 days postinfection (acute), mice were killed and salivary glands **(a)** and kidneys **(b)** examined for DNA (■) and immediate-early (IE; ■), early (E; ▨), and late (L; ▩) gene expression. Six months later, further mice were examined for MCMV DNA and RNA transcripts (latent) in salivary glands (a), kidney (b), lung **(c)**, spleen **(d)**, liver **(e)**, and heart **(f)**. Finally, at 3, 7, 14, 21 and 28 days postinitiation of immunosuppression, mice were killed and the above tissues and blood **(g)** examined similarly. The presence of a column indicates that an amplified product was obtained.

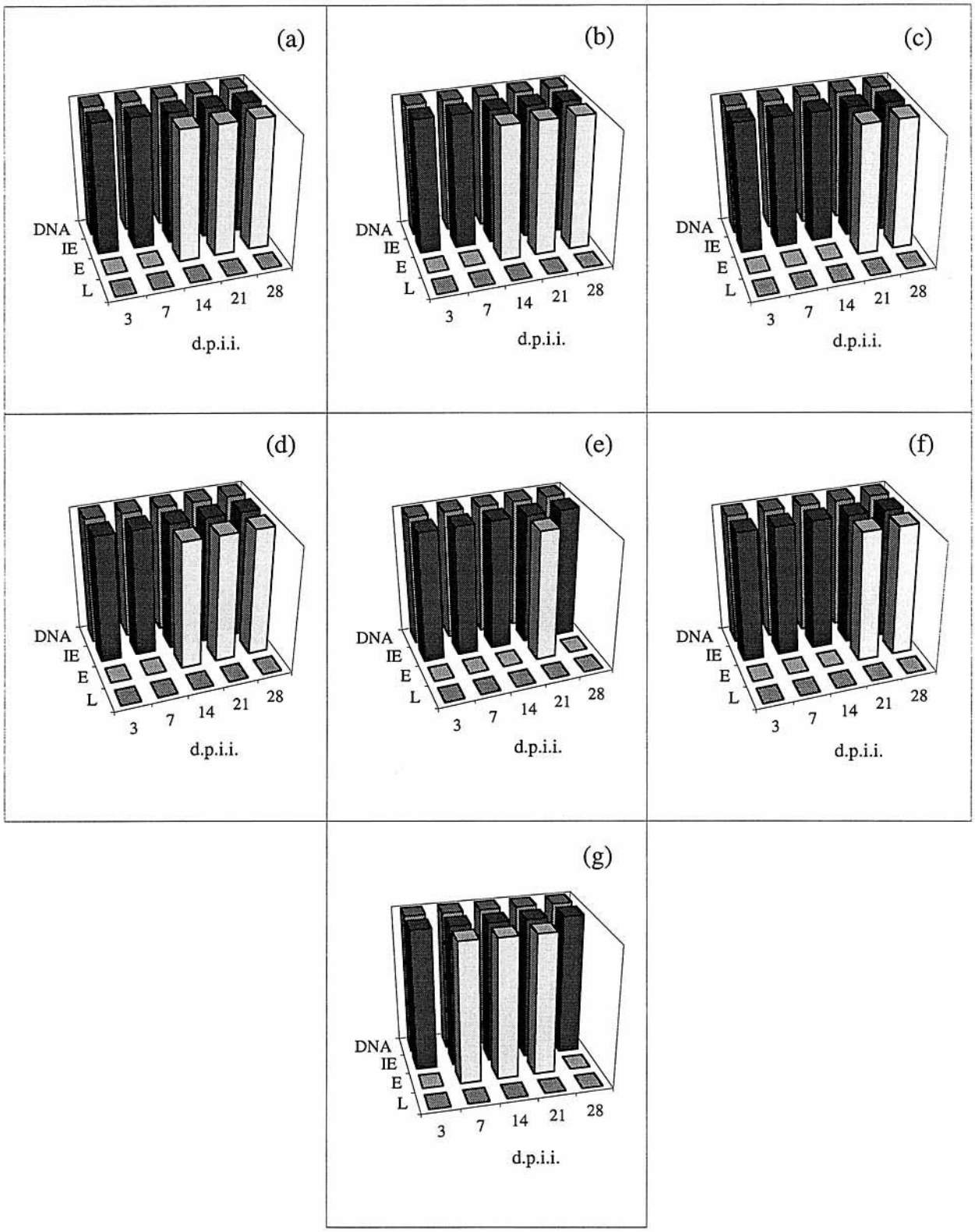

Fig. 4. PCR and RT-PCR analysis of tissues from mice infected with MCMV mutant tsm4 and reactivated by immunosuppression. At 3, 7, 14, 21, and 28 days postinitiation of immunosuppression, mice were killed and salivary glands **(a),** lung **(b),** spleen **(c),** liver **(d),** kidney **(e),** heart **(f),** and blood **(g)** examined for DNA (■) and immediate-early (IE; ■), early (E; ▨), and late (L; ▩) gene expression. The presence of a column indicates that an amplified product was obtained.

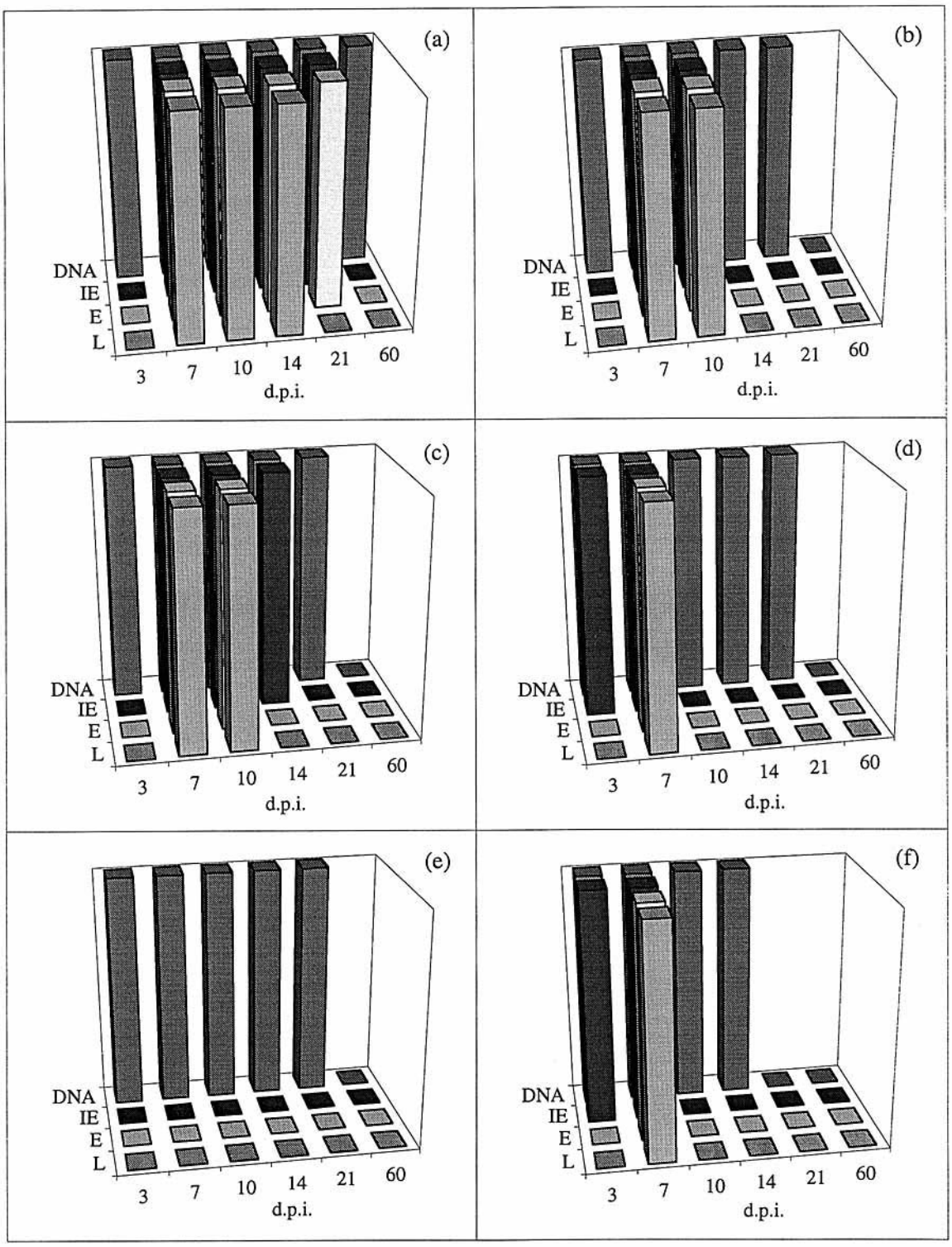

Fig. 5. PCR and RT-PCR analysis of tissues from mice infected with MCMV mutant tsm5. At 3, 7, 14, 21, and 60 days postinfection, mice were killed and salivary glands **(a)**, spleen **(b)**, lung **(c)**, liver **(d)**, kidney **(e)**, and heart **(f)** examined for DNA (■) and immediate-early (IE; ■), early (E: ▨), and late (L; ▨) gene expression. The presence of a column indicates an amplified product was obtained.

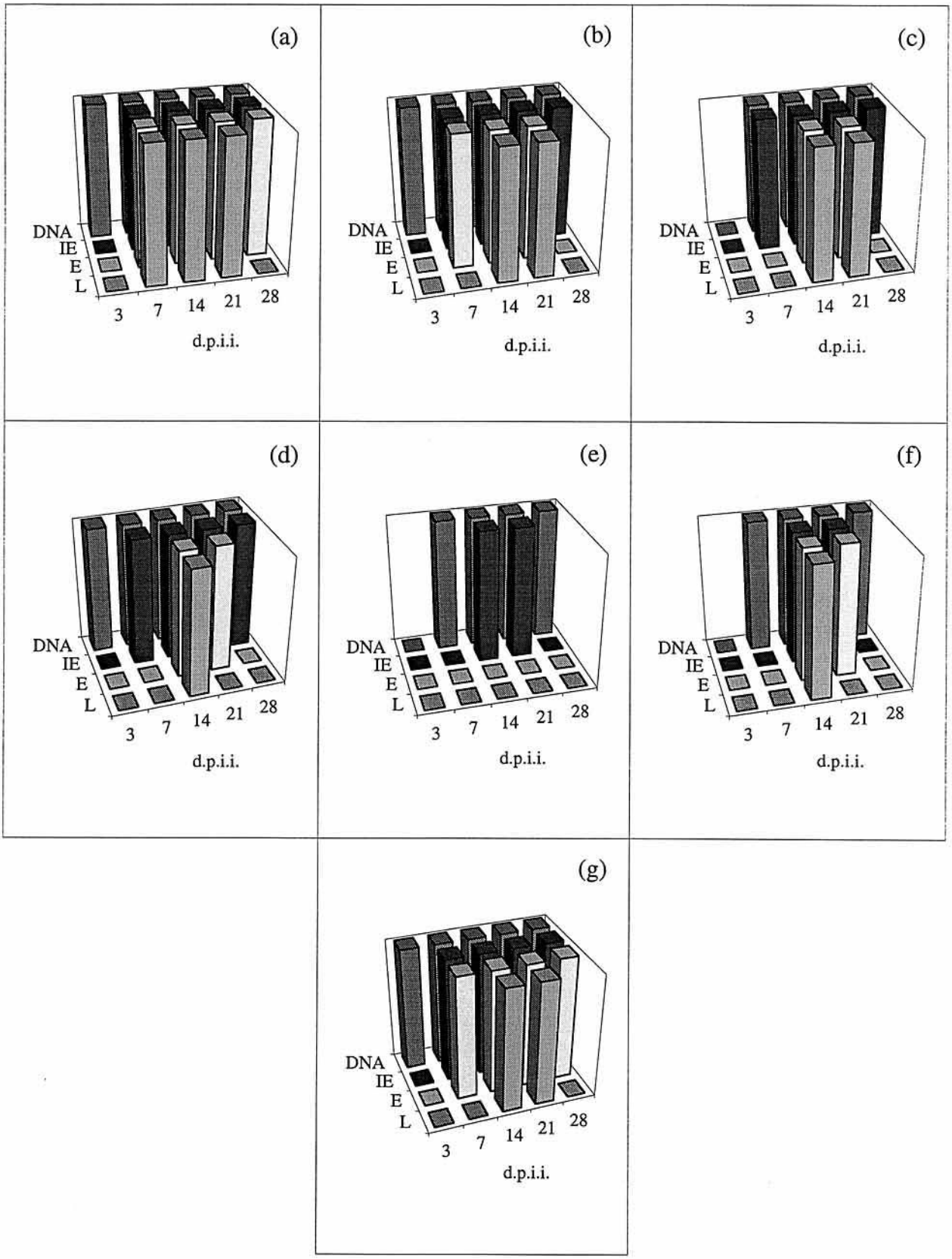

Fig. 6. PCR and RT-PCR analysis of tissues from mice infected with MCMV mutant tsm5 and reactivated by immunosuppression. At 3, 7, 14, 21, and 28 days postinitiation of immunosuppression, mice were killed and salivary glands **(a)**, lung **(b)**, spleen **(c)**, liver **(d)**, kidney **(e)**, heart **(f)**, and blood **(g)** examined for DNA (■) and immediate-early (IE; ■), early (E; ▨), and late (L; ▩) gene expression. The presence of a column indicates that an amplified product was obtained.

to be maintained in tissues of latently infected mice in a true molecular latency not as low level persistent virus. Third, genes additional to IE genes are involved in reactivation from latency.

The mechanisms by which the CMV genome enters and is maintained in the latent state is poorly understood. One interesting finding of the present studies is that it is not necessary for the virus to undergo productive infection in a tissue or cell for the virus to become latent. Mutant tsm5, which failed to produce detectable virus in any organ, nevertheless became latent. It is thus possible, since CMV infection is cytocidal and causes histological lesions in affected organs, that latency is established in nonproductively infected cells.

Some studies suggest that transcription is limited to immediate-early genes during the latent state [Saltzman et al., 1988; Schrier et al., 1985], whereas others have failed to detect any transcription [Bitsch et al., 1993]. No latency-associated transcripts similar to those for α herpesviruses have yet been defined for CMV. Recently, IE gene expression has also been detected in neurons infected with herpes simplex virus (HSV) [Kramer and Coen, 1995]. Yuhasz et al. [1994] have demonstrated MCMV DNA and IE gene expression in lungs of latently infected mice, but only DNA in spleen and bone marrow, and have suggested that lungs support a chronic persistent infection and the other organs a true latent infection. This needs to be regarded with caution, since the authors were unable to examine early and late transcriptional events because of the lack of sensitivity of their PCR assay and their inability to differentiate between the amplified products from DNA and RNA templates for genes without introns. In addition, we have detected IE gene expression in salivary glands but not lungs or spleen, whereas others [Henry and Hamilton, 1993] have detected IE expression in spleen during latency. Furthermore, IE gene expression does not necessarily indicate a low level chronic infection or transient reactivation. Although we detected IE gene expression in salivary glands, no detectable E or L gene expression was observed and no infectious virus was recoverable. Again, persistent low level virus infection could not be detected in salivary glands, spleens, or kidneys of latently infected mice even with infectivity assays capable of detecting 1–3 pfu/organ [Pollock and Virgin, 1995]. Interestingly, we detected IE gene expression only in mice latently infected with the *wt* K181 strain, but not in mice infected with any of the mutants, suggesting that IE promoter function is relatively inefficient and that the level of expression differs between viruses and tissues. It would be interestingly to examine the number of cells expressing IE transcripts in different tissues of mice infected with the different viruses using in situ hybridization.

The present results, as previous observations, suggest multiple sites of latency. Thus whole blood [Cheung and Lang, 1977a], heart [Rubin et al., 1984], arterial walls [Melnick et al., 1994], uterus [Furukawa et al., 1994], lung [Balthesen et al., 1993; Collins et al., 1993; Furukawa et al., 1994], liver [Dallas et al., 1994], spleen [Klotman et al., 1990; Mercer et al., 1988], brain [Collins et al., 1993], salivary glands [Cheung and Lang, 1977b; Klotman et al., 1990], prostate [Cheung and Lang, 1977b], and kidney [Hamilton and Seaworth, 1985] have been suggested as sites of latent infection. The cell type in which the virus remains latent is problematical. Whereas virus may be present in many organs in reactivated individuals or animals, it has not been possible to disprove that virus reactivates at one site only and spreads to other sites. However, it has been argued that blood cells such as macrophages [Brautigan et al., 1979] and B lymphocytes [Jordan et al., 1977] may be the common cell type harbouring latent virus in these organs. Similarly, HCMV has been found latently to infect human monocytes [Stanier et al., 1989; Taylor-Wiedeman et al., 1991], and indeed they may be the source of infection in transplanted kidneys [Gnann et al., 1988]. However, recent studies have suggested endothelial cells as sites of latency of HCMV in arterial walls [Hendrix et al., 1991]. Similarly, mature lymphocytes, macrophages, or dendritic cells appear not to be sites of latency in spleens of latently infected mice [Mercer et al., 1988], where it has been suggested that stromal cells are involved [Pomeroy et al., 1991]. Blood cells also appear unlikely as the site of latency in lung tissue [Balthesen et al., 1993]. The data in the present communication possibly supports the blood cell as the site of latency, since gene expression during reactivation occurred first in blood and then other organs with all viruses. In addition, some organs were MCMV DNA negative before immunosuppression, yet gene expression was evident during immunosuppression and infectious virus was produced. For example, DNA could not be detected in the kidneys of mice infected with *wt* virus immediately prior to immunosuppression, yet IE, E, and L gene expression was detected during immunosuppression and infectious virus was liberated. This expression was delayed at least 7 days relative to reactivation from blood, suggesting that virus detected in the kidney may well have originated elsewhere. Against this view is the observation that MCMV DNA and IE-1 gene expression could be detected in all tissues as early as 3 days p.i.i., suggesting that virus may reactivate at different rates in different tissues. It would be likely then that MCMV DNA is present in all tissues but not necessarily at levels detectable by PCR and that its early appearance after immunosuppression reflects cellular rather than viral replication.

In an attempt to understand the mechanism of reactivation from latency, many studies have compared CMV replication in undifferentiated or unstimulated cells in vitro with their differentiated or stimulated counterparts to serve as a model of latency and reactivation [see Mocarski, 1993; Stinski et al., 1993 for reviews]. These studies seem to indicate that once the major immediate early promoter is activated by host or viral transcription factors, productive infection ensues. Studies with herpes simplex virus (HSV) also have led investigators to suggest that reactivation is the inevitable consequence once IE gene expression has been initiated [Kemp and Latchman, 1989; Minagawa et al., 1994]. In contrast, a

new model of HSV latency in ganglionic neurons suggests that reactivation occurs in stages [Kramer and Coen, 1995]. Most latently infected cells express IE RNA at levels insufficient for E gene expression. However, when perturbed during reactivation, IE gene expression may be upregulated such that some E gene products may be synthesized. Some cells may accumulate sufficient E RNAs to threshold levels to permit DNA replication and some L gene expression. Such DNA replication and/or L gene products may then upregulate IE gene expression, leading to the lytic cascade and production of viral particles [Kramer and Coen, 1995]. This is supported by studies on CMV replication in monocytes of healthy carriers [Taylor-Wiedeman et al., 1991]. Such monocytes contain MCMV DNA but no expression of early or late genes occurs in vitro until they are stimulated with granulocyte colony stimulating factor and hydrocortisone. Then, immediate-early and delayed-early, but not late, gene transcription was evident, and this did not result in a productive infection. Our observations in mice with tsm4 also support this model, since expression of IE-1, E-1 and gB genes with productive infection occurred during acute infection, but only IE-1 and E-1, but not gB genes were expressed during reactivation, with E-1 gene expression being transitory in some tissues. This suggests that IE gene expression is limited and that either DNA replication does not occur or the late gene(s) required for upregulation of IE gene expression to levels sufficient for productive infection are not produced. Interestingly, tsm4 is a late function defective mutant at its nonpermissive temperature of 39°C, suggesting that the ts gene may be involved in the defect in reactivation even at the normal temperature of the mouse (36–37°C).

With tsm5 IE, E, and L gene expression occurred in all tissues except kidney during reactivation as during acute infection and in both acute and reactivated infection, no detectable levels of infectious virus were evident. It seems likely therefore that the block is similar during acute infection and reactivation with this mutant and that the mutated gene plays little or no role in the reactivation process. We are attempting to identify the ts genes in both mutants and have shown that the ts phenotype of both may be rescued by the 27.4 kbp *Hin*dIII B fragment of *wt* DNA (unpub. obs.).

A final intriguing observation is the presence of IE, E, and L transcripts of tsm5 in all tissues examined during acute infection despite the lack of a detectable productive infection in any tissue. How does virus spread from the inoculation site to these organs? Recently, it has been shown, using LacZ tagged virus, that mononuclear phagocytes are the vehicle of dissemination from the peritoneal cavity to other organs during acute infection [Stoddart et al., 1994]. Whereas it is generally accepted that such mononuclear cells do not support the complete viral reproductive cycle, they nevertheless can transmit infection to other cell types such as vascular endothelial cells [Waldman et al., 1995]. Such mononuclear phagocytes may thus be the vehicle for transport and transmission of tsm5 to various organs.

Prophylaxis of CMV infection by vaccination with live virus vaccine has been confounded by the problem of potential reactivation of the vaccine virus, possibly causing life-threatening complications. Tsm4 and tsm5 present possibilities for circumventing these problems, permitting the development of live vaccines. Tsm4 is capable of productive primary infection and should therefore elicit a normal immune response. However, the virus does not progress to productive infection from latency under normal reactivating conditions and is therefore unlikely to cause any CMV associated disease during reactivation. In contrast, tsm5 does infect target cells but never develops a productive infection, developing something like a permanent latent infection. This may be sufficient to elicit a protective immune response, the latent virus may indeed competitively inhibit further CMV infections. Determination of the ts genes in tsm4 and tsm5 may allow identification of the HCMV homologous genes allowing for the production of safer live vaccines.

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